

## Functional Studies of P-glycoprotein in Inside-out Plasma Membrane Vesicles Derived from Murine Erythroleukemia Cells Overexpressing MDR 3

PROPERTIES AND KINETICS OF THE INTERACTION OF VINBLASTINE WITH P-GLYCOPROTEIN AND EVIDENCE FOR ITS ACTIVE MEDIATED TRANSPORT\*

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Active [<sup>3</sup>H]vinblastine (VBL) transport (efflux) was documented for inside-out plasma membrane vesicles from murine erythroleukemia cells (MEL/VCR-6) resistant to vinca alkaloids and overexpressing MDR 3 P-glycoprotein (P-gp) 80-fold. Uptake of [<sup>3</sup>H]VBL at 37 °C by these inside-out vesicles, but not rightside-out vesicles or inside-out vesicles from wild-type cells, was obtained in the form of a rapid, initial phase (0–1 min) and a slower, later phase (>1 min). The rapidity of each phase correlated with relative P-gp content among different MEL/VCR cell lines. The initial MDR-specific phase was temperature- and pH-dependent (optimum at pH 7), osmotically insensitive, and did not require ATP. The second MDR-specific phase was temperature-dependent, osmotically sensitive, and strictly dependent upon the presence of ATP ( $K_m = 0.37 \pm 0.04$  mM). Although other triphosphate nucleotides were partially effective in replacing ATP, the nonhydrolyzable analogue ATP $\gamma$ S (adenosine 5'-O-(thiotriphosphate)) was ineffective. This time course appears to represent tandem binding of [<sup>3</sup>H]VBL by P-gp and its mediated transport, with the latter process representing the rate-limiting step. In support of this conclusion, both binding and transport were inhibited by verapamil, quinidine, and reserpine, all known to be inhibitors of photoaffinity labeling of P-gp, but only transport was inhibited by C219 anti-P-gp antibody or orthovanadate. Although the rate of transport of [<sup>3</sup>H]VBL was 7–7.5-fold lower than the rate of binding ( $V_{max} = 104 \pm 15$  pmol/min/mg protein,  $K_m = 1.5 - 2 \times 10^5$  mol<sup>-1</sup> s<sup>-1</sup>) to P-gp, each phase exhibited saturation kinetics and values for apparent  $K_m$  and  $K_D$  for each process were approximately the same (215  $\pm$  35 and 195  $\pm$  30 nM). Intravesicular accumulation of [<sup>3</sup>H]VBL was almost completely eliminated by high concentrations of nonradioactive VBL, suggesting that simple diffusion does not contribute appreciably to total accumulation of [<sup>3</sup>H]VBL in this vesicle system. This could be at least partially explained by the fact that these inside-out vesicles under the conditions employed did not maintain a P-gp mediated pH gradient. However,

ATP-dependent, intravesicular accumulation of osmotically sensitive [<sup>3</sup>H]VBL occurred against a substantial permeant concentration gradient in both a time- and concentration-dependent manner consistent with an active, saturable process.

Multidrug resistance is a phenomenon in which the acquisition of resistance by tumor cells to a cytotoxic agent concurrently results (1–6) in resistance to other cytotoxic agents that are structurally dissimilar. One common form of multidrug resistance seen both in rodent and human tumor cells is a consequence (1–12) of overexpression of one or more homologs of a membrane glycoprotein, P-glycoprotein (P-gp),<sup>1</sup> that is associated with ATP-dependent egress of these cytotoxic drugs from drug-resistant tumor cells. Although most studies of multidrug resistance have focused (7–12) on model tumor cell systems *in vitro*, there is also evidence of its manifestation in model systems *in vivo* (13, 14) and in neoplastic disease in patients (15, 16). A considerable number of studies have been carried out on both the biochemical properties of P-gp (1–12) and on the molecular genetics related (1–12) to its gene expression in multidrug-resistant cells. These studies have identified P-gp as a member of a family of ATP-binding membrane transporters found in a variety of procaryotic and eucaryotic cells. Despite these very detailed and informative studies, the precise mechanism by which P-gp mediates multidrug resistance is not fully understood. In fact, a number of controversies have recently emerged (17–24) with regard to the nature of the mechanism, the interaction of P-gp with ATP, and the putative role of P-gp as a direct mediator of outward transport.

One methodologic approach that has yielded meaningful information on the function of P-gp in a variety of cell types incorporates membrane vesicle technology. Such studies of P-gp utilized mixed populations of inside-out *versus* rightside-out plasma membrane vesicles (25–30) or populations enriched (31, 32) for inside-out vesicles. Some particularly innovative approaches have recently been described. One utilized enriched inside-out plasma membrane vesicles containing P-gp derived from yeast cells (33) or their secretory vesicles (34) stably transfected with mouse *mdr 3* cDNA. The other reconstituted (35) the hamster P-gp homologue of mouse MDR 3 P-gp in artificial proteoliposomes. All of these studies documented temperature-

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<sup>1</sup> The abbreviations used are: P-gp, P-glycoprotein; ATP $\gamma$ S, adenosine 5'-O-(3-thiotriphosphate); MDR (*mdr*), multidrug resistance; MEL, murine erythroleukemia; MOPS, 3-(*N*-morpholino)propanesulfonic acid; VBL, vinblastine; VCR, vincristine; VRP, verapamil; CFCCP, carboxyl cyanide *p*-(trifluoromethoxy)phenylhydrazine, 2,4-DNP, 2,4-dinitrophenol; QND, quinidine; RSP, reserpine.

dependent, osmotically sensitive intravesicular accumulation of one or more of the multidrug-resistant related cytotoxic agents that appeared to be dependent upon ATP hydrolysis. They also showed that the intravesicular accumulation of these cytotoxic drugs was perturbed by a multidrug resistance modulator, namely VRP, or by an anti-P-gp antibody.

Although highly informative, the above studies provided little or no direct evidence for P-gp-mediated active transport in the form of concentrative accumulation of these agents in an osmotically active intravesicular fraction. In the studies described here, we applied membrane vesicle technology to an examination of the function of P-gp in murine erythroleukemia (MEL) cells overexpressing only MDR 3. The methodology employed was similar to that we had used for our earlier studies (35) of ATP-dependent efflux of folate compounds in inside-out plasma membrane vesicles from L1210 cells. In addition to our own characterization of this process, using [<sup>3</sup>H]VBL as the permeant, we provide kinetic evidence for a delineation of the rate of binding to P-gp from the rate of intravesicular accumulation and showed within the same time-course experiment that both events did appear to proceed tandemly with the latter process being the rate-limiting step. We also obtained direct evidence for active P-gp-mediated efflux by demonstrating that intravesicular accumulation of osmotically active [<sup>3</sup>H]VBL occurs against a concentration gradient in these vesicles. To adequately address this issue, we believed that it was first necessary to 1) determine the degree of purity and sidedness (inside-out versus rightside-out) of the membrane vesicle preparation, 2) quantitate the inside-out intravesicular volume per unit of plasma membrane protein, and 3) delineate the osmotically active fraction of ATP-dependent intravesicular accumulation of [<sup>3</sup>H]VBL from the non-osmotically active fractions of [<sup>3</sup>H]VBL associating specifically (P-gp) or nonspecifically with the vesicles. Earlier studies (25–34) did not adequately address this issue in that only some, but not all, of these measurements were actually carried out. The results of our studies are described below. A preliminary report of these studies was recently presented (37) in abstract form.

#### EXPERIMENTAL PROCEDURES

**Cell Growth and Isolation.**—Multidrug-resistant and parental MEL cells for vesicle preparations were obtained as ascites suspensions from cyclophosphamide-treated (100 mg/kg 1 day prior to implant) BD2F1 hybrid (C57BL × DBA/2b1) mice following adaption to growth in DBA/2b mice. Mice bearing multidrug-resistant cells were treated twice weekly with the maximum tolerated dose of VCR. Parental MEL cells (strain SC-9) and VCR-resistant MEL cells used in these studies were derived from a VCR-resistant MEL cell line (MEL/V3-17) obtained from Dr. June L. Biedler. Parental and resistant MEL cells were also grown as suspension cultures in  $\alpha$ -minimal essential medium/Eagle's F-12 (1:1) medium plus 5% fetal calf serum. Derivation of additional multidrug-resistant cell lines of higher level of resistance from MEL/V3-17 cells was carried out by stepwise selection in higher concentrations of VCR during serial transfer and cloned by limiting dilution.

**Northern Blot Analyses.**—Poly(A)<sup>+</sup> RNA was extracted from MEL cells on an oligo(dT) column and evaluated for its integrity by a previously reported (38) procedure. An aliquot of the same RNA was analyzed by Northern blotting using truncated mouse mdr 1 and mdr 3 cDNA (39) as a probe (generously provided by Dr. Philippe Gros, McGill University, Montreal, Canada) and normalized to  $\gamma$ -actin RNA content with a human  $\gamma$ -actin probe, PCD-2-actin (40). Labeling of each probe was by random priming (Random Primers DNA labeling kit, Boehringer Mannheim) using [<sup>32</sup>P]dCTP (3000 Ci/mmol) and 100 ng of insert. The hybridization signals were monitored by radioautography and (or) quantitated by means of a Betagen 603 Blot analyzer (Betagen) correcting for nonspecific background in a parallel blank blot.

**Southern Blot Analyses.**—Genomic DNA from parental and resistant (MEL/VCR-6) frozen cell pellets was prepared (41) and digested with *Eco*RI. The DNA was separated by electrophoresis through a 0.82% agarose gel and transferred to Nytran<sup>+</sup> (Schleicher & Schuell). MDR 1 and 3 cDNA probes, hybridization, and labeling procedures were the

same as that used above.

**Immunoblotting Procedure.**—Plasma membrane preparations were electrophoresed (42) on a 7.5% polyacrylamide gel and transferred to nitrocellulose using a Bio-Rad Trans-Blot cell (42). Western blotting was performed using the C-219 monoclonal antibody (Centocor) at a concentration of 0.5  $\mu$ g/ml as the primary antibody, and anti-mouse horseradish peroxidase-IgG conjugate (Sigma) at a 1:3000 dilution as the secondary antibody (43). The blots were used to expose Hyperfilm after incubation with enhanced chemiluminescence (ECL) reagents (Amersham Corp.). Differences in P-gp expression levels were quantitated using a Stratagen 7000 densitometer. Western blots with anti-MDR 1 and anti-MDR 3 isoform-specific antibodies (39, 44), which were generous gifts from Doctors J. Croop and P. Gros, were done at 1:100 and 1:500 dilutions, respectively. Goat anti-rabbit horseradish peroxidase-IgG conjugated antibody was used at a 1:4000 dilution and visualized as above using ECL.

**Plasma Membrane Vesicle Preparation.**—The procedure employed for inside-out vesicles has been described in detail (36) as a modification of the method of Marin *et al.* (45), which was originally adapted (36) to L1210 cells by us in accordance with the review by DePieris and Karnovsky (46). Vesicles were stored in transport buffer (100 mM MOPS, pH 7, 125 mM, sucrose and 5 mM MgCl<sub>2</sub>) on ice until used. Approximately 1 mg of inside-out vesicles were obtained from 2–2.5  $\times 10^8$  cells. Intravesicular volume of the inside-out vesicles prepared from parental or multidrug-resistant cells, assuming 100% sidedness in this orientation, was 1.6  $\pm$  0.3  $\mu$ l/ $\mu$ g protein ( $n = 4$ ) as determined by a standard procedure (47). Vesicles that were 100% in the rightside-out plasma membrane vesicles were prepared by 5-fold dilution of the vesicle preparation in H<sub>2</sub>O, which was vigorously shaken, then centrifuged at 1400  $\times g$  for 10 min and resuspended in transport buffer.

**Vesicle Sidedness and Contamination Markers.**—The orientation of the vesicles was determined by ecto enzyme (alkaline phosphatase, EC 3.1.3.1) and endo enzyme (glyceraldehyde-3-phosphate dehydrogenase, EC 1.2.1.12) determinations (36) in the presence and absence of 0.2% (v/v) Triton X-100. The purity of the vesicle preparations as determined from an assessment (36) of various organelle-specific enzyme markers and a protein determination (48) modified according to Peterson (49) was 94–96% plasma membrane.

**Transport Experiments.**—The procedure employed is similar to that used in our prior studies (36) on ATP-dependent efflux of folates analogues by L1210 cell plasma membrane vesicles. Twenty- $\mu$ l aliquots of vesicles (50–70  $\mu$ g of membrane protein) were preincubated for 30 s at 37 °C with 30  $\mu$ l of transport buffer with or without a regenerating system (31) in siliconized glass tubes. The reaction was started by the addition of [<sup>3</sup>H]VBL and ATP to the tubes containing regenerating system and the addition of [<sup>3</sup>H]VBL alone to tubes without regenerating system. After the required time interval, the reaction mixture was diluted with 9 ml of ice-cold medium, the vesicles collected by filtration in HAWP 0.45  $\mu$ m filters (Millipore) pretreated with VBL and washed three times with 9 ml of cold Medium 3. After the filters dried, they were placed in scintillation fluid for radioactive counting. The data generated were corrected for nonspecific absorption of [<sup>3</sup>H]VBL to the vesicle surface and to the filter, by a brief (5 s) incubation of vesicles at 0 °C with [<sup>3</sup>H]VBL and processing these and the [<sup>3</sup>H]VBL in transport medium alone by filtration. Each time point was carried out in duplicate, and graphed data points represent an average of at least three separate determinations done on different days.

**Other Analytic Procedures.**—Proton gradients ( $\Delta$ pH) were measured (50, 51) by partitioning of [<sup>14</sup>C]methylamine between the intervesicular and intravesicular (47) space. Any gradient in protons maintained by the vesicle preparation would increase partitioning of the protonated amine in the direction of the lower pH. [<sup>14</sup>C]methylamine (100 nM) was added to the vesicle preparation in transport buffer and incubated for 10 min at 37 °C. The vesicles were centrifuged and radioactivity in the pellet determined by scintillation counting with appropriate corrections (47).

**Chemicals.**—[<sup>3</sup>H]VBL was obtained from Amersham Biochemicals and was repurified to >98% by high performance liquid chromatography (52) every 3–4 weeks. Tris-ATP, phosphocreatine, and creatine phosphokinase were obtained from Sigma. All other chemicals were reagent-grade.

#### RESULTS

**Isolation and Characterization of Multidrug-resistant MEL Cells.**—Northern blot analysis of mRNA from MEL/VCR cells strain S(-9, Ref. 53) (MEL/VCR: 0.2–6) with mdr 1- and mdr 3-specific cDNA probes at various stages of selection showed

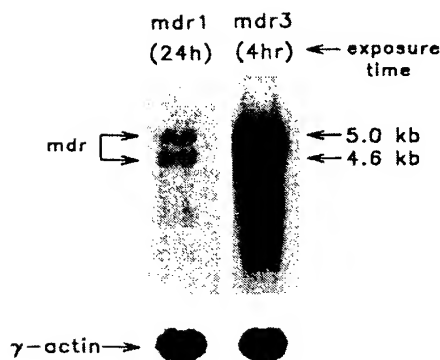


FIG. 1. Northern blot of mRNA from wild-type MEL and MEL/VCR-6 cells. The blots were hybridized with *mdr* 1 and *mdr* 3-specific cDNA probes under standard hybridization conditions. The radioactivity associated with *mdr* 1 and *mdr* 3 hybridization signals was measured by radioautography. The radioactivity associated with the  $\gamma$ -actin control hybridization signals were measured by means of the Betagen 603 blot analyzer.

(data given only for MEL/VCR-6 in Fig. 1) some degree of overexpression of both *mdr* 1- and *mdr* 3-specific mRNA. In each case, both the 4.6- and 5.0-kilobase *mdr* 1 and *mdr* 3 mRNA species, that are characteristic (54) of murine tumor cells, were equally overexpressed in these MEL cells. However, greater expression of *mdr* 3 compared to *mdr* 1 occurred consistently in an increasing manner during the selection process. When this blotting was quantitatively analyzed in the case of MEL/VCR-6 by means of the Betagen 603 analyzer, the results showed (data not given) that relative overexpression in MEL/VCR-6 cells was 98% *mdr* 3-specific and only 2% *mdr* 1-specific. Southern blot analysis of *Eco*RI-restricted genomic DNA from parental MEL (SC-9) cells and MEL/VCR-6 cells revealed (data not shown) only a modest increase (4–6-fold) in gene copy number in the resistant cells when blotted with the *mdr* 3-specific probe. In confirmation of the results from the Northern blotting, Western blotting of MEL/VCR-0.2 and MEL/VCR-6 plasma membrane protein with anti-MDR 1 and -MDR 3 peptide antibodies showed (Fig. 2) a barely visible 140-kDa band with the former and a prominent 140-kDa band with the latter using anti-MDR 3, but no discernible band using anti-MDR 1. Densitometric analysis of other blots made with C219 anti-P-gp antibody shown in Fig. 2, in which we compared wild-type to MEL/VCR-0.2 and MEL/VCR-0.2 to MEL/VCR-0.4, -0.8, -3, and -6, indicated that P-gp was overexpressed approximately 80-fold at the level of resistance of MEL/VCR-0.8 and above when compared to wild-type. The level of P-gp in these cells was similar to the level of P-gp (170 kDa) in the multidrug-resistant Chinese hamster cell line, DC-3F/ADX (Fig. 2), shown for comparison. Finally, we also found that MEL/VCR-6 cells, exhibited a pattern of cross-resistance characteristic (39) of murine tumor cells expressing MDR 3. Thus, these cells were not cross-resistant to methotrexate but were cross-resistant to actinomycin D (84-fold), doxorubicin (250-fold), colchicine (312-fold), VBL (390-fold), and VCR (3882-fold).

**Membrane Sidedness and Other Preliminary Considerations**—The addition of 0.2% Triton X-100 to the inside-out plasma membrane vesicle preparations from parental cells increased the activity of the ecto marker 76-fold but increased the activity of the endo marker only 2–3-fold. The same markers (see “Experimental Procedures”) were increased 2.5- and 1.5-fold in detergent-treated inside-out vesicles from MEL/VCR-6 cells. Calculation (36) of the sidedness from this data showed that 98% of the parental-derived vesicles and 50–60% of the MEL/VCR-6-derived vesicles were in the inside-out orientation.

Membrane marker analysis of the same plasma membrane preparation from MEL/VCR-6 cells following disruption by dilution in  $H_2O$  (see “Experimental Procedures”) and revesiculation showed (data not given) that the reformed vesicles were almost 100% in the rightside-out orientation.

Possible complications associated with the contribution to total intravesicular accumulation of influx of [ $^3H$ ]VBL in the 40–50% of the vesicle preparation derived from MEL/VCR-6 cells remaining rightside-out were avoided during the course of these studies because of other properties of MEL/VCR. Similar to other tumor cells (55) selected for resistance to vinca alkaloids, influx of [ $^3H$ ]VBL in parental cells was found (data not shown) to be 20-fold lower than in the resistant cells. Finally, since any proton gradient maintained by the vesicle preparations used in these studies might appreciatively influence our results, we sought to ascertain whether such a gradient does, in fact, exist with the buffer used that contained 50 mM of available protons. To do this, we measured the partitioning of [ $^{14}C$ ]methylamine between the intervesicular and intravesicular space during incubation of 100 nM of the amine with the inside-out vesicle preparation from MEL/VCR-6 cells in transport buffer with and without ATP and found that it never exceeded unity (data not shown). We also controlled for partitioning of the amine in the rightside-out vesicle compartment by the same procedure. With a 100% rightside-out preparation derived from the same cell line we obtained the same result (unity).

**ATP Dependence for Intravesicular Accumulation of [ $^3H$ ]VBL**—The results given in Fig. 3A for a typical time course for [ $^3H$ ]VBL uptake in vesicles from MEL/VCR-6 cell show that little uptake was observed at 0 °C either in the presence or absence of 5 mM ATP. In contrast, rapid uptake occurred at 37 °C in the presence of ATP. Also, uptake at this temperature was bimodal with respect to time. The rate was more rapid initially but was 7–7.5-fold lower after 30 s of incubation. Although only the time course for one concentration (70 nM) used in this experiment is shown, the same bimodal time course was obtained (data not shown) at higher or lower concentrations of [ $^3H$ ]VBL. In the absence of ATP, uptake at 37 °C exhibited only a rapid initial phase with cessation of uptake after 1 min. Under the assumption (see following sections) that the complex time course obtained in the presence of ATP represented specific binding to P-gp followed by P-gp-mediated internalization of [ $^3H$ ]VBL into the intravesicular compartment, we determined the actual rate of P-gp binding of [ $^3H$ ]VBL. This was obtained by “subtracting out” the later, slower phase, which was quantitated by the back-extrapolation shown in the figure (Fig. 3A), from total uptake. This data replotted in Fig. 4B shows that the rate of initial uptake ( $9.8 \pm 1.5$  pmol/min/mg protein) was the same in the presence or absence of ATP. In addition, VRP, as well as QND and RSP (data not shown), were potent inhibitors of this initial uptake, while the addition of 100  $\mu$ M VBL virtually eliminated uptake. As shown in Fig. 4, after the rapid, initial period of uptake, ATP-dependent uptake of [ $^3H$ ]VBL was linear with time over the 1–10-min interval of incubation employed. Again, essentially no uptake of [ $^3H$ ]VBL occurred with these vesicles in the absence of ATP (Fig. 4A) beyond the first 30 s of incubation. Also, there was no significant vesicular accumulation of [ $^3H$ ]VBL at 37 °C by inside-out vesicles derived from wild-type MEL cells (Fig. 4A) or rightside-out vesicles derived from MEL/VCR-6 cells (data not shown). Other data are also shown (Fig. 4B) in this figure on the inhibition by VRP of ATP-dependent uptake of [ $^3H$ ]VBL at 37 °C. In addition to the reduction in the slope of the 1–10-min time course obtained in the presence of 10  $\mu$ M VRP, the back-extrapolation of the time course to the origin shows a down-

## A. C-219 MONOCLONAL ANTIBODY

## B. ISOFORM SPECIFIC ANTIBODIES

Fig. 2. Western blot analyses of P-gp in plasma membrane from wild-type and multidrug-resistant MEL cells. Plasma membrane was solubilized in detergent prior to SDS-polyacrylamide gel electrophoresis. A, blot of wild-type and resistant MEL cells with C219 antibody. B, blot of wild-type MEL cells and MEL/VCR-0.2 and MEL/VCR-6 cells with anti-MDR 1 and anti-MDR 3 P-gp antibodies. Detection of antibody-specific signals was by enhanced chemiluminescence (ECL) detection. The figure depicts the results of typical analyses.

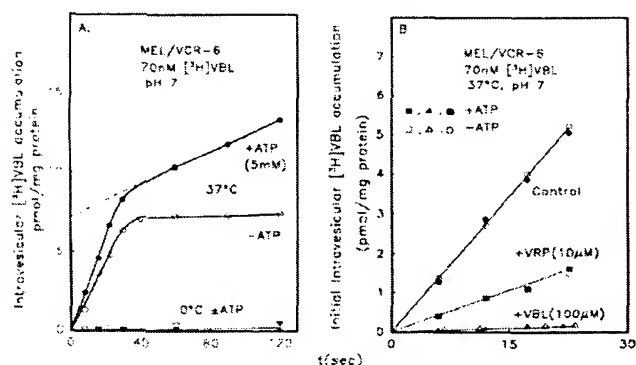
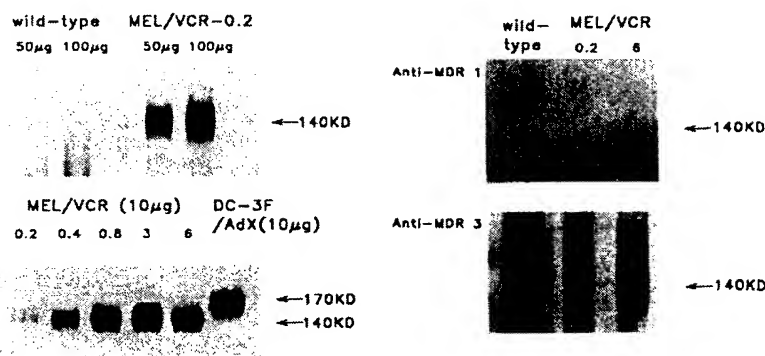


Fig. 3. Time course for uptake of  $[^3\text{H}]\text{VBL}$  by inside-out vesicles from MEL/VCR-6 cells. Vesicles were incubated with 70 nM  $[^3\text{H}]\text{VBL}$  in transport buffer for the times indicated. A, initial and later linear phases of uptake of  $[^3\text{H}]\text{VBL}$  by vesicles at 37 °C and 0 °C in the presence and absence of ATP. B, the true rate of the initial phase of uptake of  $[^3\text{H}]\text{VBL}$ . Additional experimental details are provided in the text. Data are an average of three experiments. Standard error of the mean =  $\pm 13\%$ .

ward dislocation. Such a result is consistent with the effect of VRP on the rapid, initial phase of uptake of  $[^3\text{H}]\text{VBL}$  shown in Fig. 3B. In contrast, the effect of C219 antibody is quite different (Fig. 4B). Although there was a reduction in the slope of the time-course plot for  $[^3\text{H}]\text{VBL}$  uptake in its presence, there was no downward dislocation of the back-extrapolated time-course plot. We also observed during these studies that measurement of  $[^3\text{H}]\text{VBL}$  uptake during longer term incubations of these inside-out vesicles were not possible either because of the instability of the vesicles or the accumulation of inhibitory end products of the regenerating system.

Other data were obtained in experiments measuring uptake of  $[^3\text{H}]\text{VBL}$  at 37 °C by inside-out vesicles in different concentrations of sucrose, which modulates intravesicular volume. These data, given in Fig. 5, show that uptake of  $[^3\text{H}]\text{VBL}$  obtained within 30 s of incubation was unaffected by differences in sucrose concentration and, thus, was osmotically insensitive, while a portion of the uptake of  $[^3\text{H}]\text{VBL}$  measured after 2 min of incubation or beyond (data not shown) was osmotically sensitive. Moreover, in the absence of ATP, no osmotically sensitive fraction of  $[^3\text{H}]\text{VBL}$  uptake could be demonstrated (data not shown). Thus, consistent with the other data already presented, these results would appear to identify the rapid initial phase of the uptake time course at 37 °C as a binding event and the slower, latter phase as mediated intravesicular accumulation (transport).

Kinetics of ATP and  $[^3\text{H}]\text{VBL}$  Concentration Dependences—

Since rate measurements constant with time could be derived for both initial and later phases in the presence of ATP, a valid kinetic analysis of these phases of uptake was possible. The effect of different ATP concentrations on the rate of osmotically active uptake (second phase) of  $[^3\text{H}]\text{VBL}$  (external concentration = 50 nM) by inside-out membrane vesicles from MEL/VCR-6 cells is shown in Fig. 6. The response curve given shows saturation kinetics in the concentration range of 0–5 mM ATP. From the double-reciprocal plot of these data also given in the figure, a single saturable component was derived with an apparent  $K_m$  of  $0.37 \pm 0.4$  mM and  $V_{max}$  of  $3.2 \pm 0.5$  pmol/min/mg protein.

The results of other experiments showed (data not given) that ATP could be replaced by either GTP or CTP as an activator of intravesicular accumulation of  $[^3\text{H}]\text{VBL}$ . However, in this case, these other triphosphonucleotides when added at 1 mM were only one-fourth as active as ATP. We also showed that the same concentration of ATPyS was without effect as an activator of intravesicular accumulation of  $[^3\text{H}]\text{VBL}$  but was an inhibitor ( $IC_{50} = 0.7$  mM) of ATP-dependent accumulation. Similarly, orthovanadate was a potent inhibitor ( $IC_{50} = 7$  mM) of ATP-dependent intravesicular accumulation of  $[^3\text{H}]\text{VBL}$ .

In other experiments, the  $[^3\text{H}]\text{VBL}$  concentration dependence values for the rate of initial and later phases of ATP-dependent intravesicular accumulation of this vinca alkaloid at 37 °C were obtained with inside-out vesicles from MEL/VCR-6 cells. The two concentration-response curves obtained in the presence of 1 mM ATP are presented as a double-reciprocal plot in Fig. 7. In each case, a single saturable component was delineated. The values for  $V_{max}$  derived from this data were  $104 \pm 15$  pmol/min/mg protein for the initial phase and  $14.3 \pm 1.8$  pmol/min/mg protein for the later, osmotically active phase. Interestingly, the value for  $K_d$  for putative (initial phase) binding and apparent  $K_m$  for transport were approximately the same,  $195 \pm 30$  and  $215 \pm 35$ , respectively.

**pH Dependence for Putative Binding of  $[^3\text{H}]\text{VBL}$  to P-gp—**The initial rate of accumulation of  $[^3\text{H}]\text{VBL}$  by MEL/VCR-6-derived vesicles at 37 °C was determined at several different pH values. The initial rate was measured with 29 nM  $[^3\text{H}]\text{VBL}$  as permeant in the presence and absence of 5 mM ATP and regenerating system. The data are given in Fig. 8 and show a single optimum for binding at pH 7 with substantial reduction in the rate of binding at higher or lower pH. The same pH optimum and profile were obtained (data not shown) for this uptake phase in the absence of ATP.

**Evidence for Osmotically Sensitive Intravesicular Accumulation of  $[^3\text{H}]\text{VBL}$  Against a Concentration Gradient—**In the experiments described above, reproducible measurements of in-

Fig. 4. Time course for uptake of [ $^3$ H]VBL by inside-out vesicles from wild-type MEL and MEL/VCR-6 cells. The data depict the second of two linear phases of uptake with 70 nM [ $^3$ H]VBL for each cell type in the presence and absence of ATP. A, comparison of the time course for uptake of [ $^3$ H]VBL  $\pm$  ATP in vesicles derived from wild-type and resistant cells. B, comparison of the effects of VRP and C219 antibody. Additional experimental details are provided in the text. Data are an average of three experiments. Standard error of the mean =  $\leq 15\%$ .

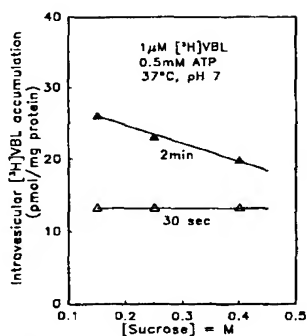
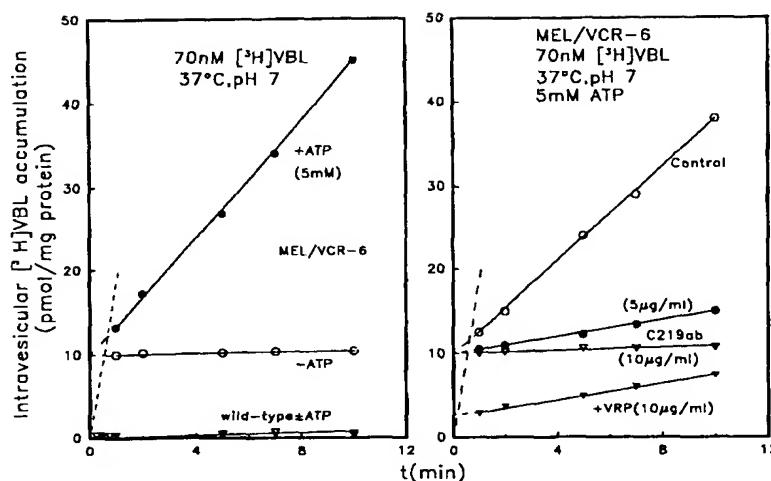


Fig. 5. Osmotic sensitive and insensitive uptake of [ $^3$ H]VBL by inside-out vesicles from MEL/VCR-6 cells. Uptake of [ $^3$ H]VBL at an external concentration of 1  $\mu$ M was measured at 37  $^{\circ}$ C over different times in the presence of varying concentrations of sucrose. Additional experimental details are provided in the text. Standard error of the mean is  $\leq 12\%$  for three separate experiments.

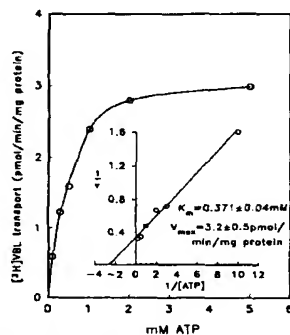


Fig. 6. Kinetics of ATP concentration dependence for intravesicular accumulation of [ $^3$ H]VBL (second phase) by inside-out vesicles prepared from MEL/VCR-6 cells. The rate of [ $^3$ H]VBL uptake at 37  $^{\circ}$ C beyond the first minute was determined at different concentrations of ATP. Inset, double-reciprocal plot of the concentration-response data. The concentration of [ $^3$ H]VBL was 50 nM in three separate experiments. Additional experimental details are provided in the text. Standard error of the mean =  $\leq 13\%$ .

travesicular volume and sidedness and the osmotically active fraction of [ $^3$ H]VBL associated with inside-out vesicles from MEL/VCR-6 cells were readily obtained. With all of these parameters in place, we were able to quantitate from the difference in ATP-dependent and -independent uptake the concentration of osmotically sensitive [ $^3$ H]VBL within the inside-out

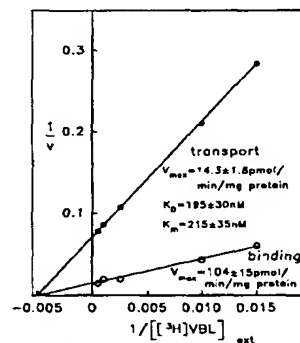


Fig. 7. Kinetics of [ $^3$ H]VBL concentration-dependence for ATP-dependent intravesicular accumulation of [ $^3$ H]VBL (initial and second phases) by inside-out vesicles from MEL/VCR-6 cells. The rate of uptake of [ $^3$ H]VBL at 37  $^{\circ}$ C within 1 min and within a 1–10-min interval was measured with different concentrations of [ $^3$ H]VBL. The concentration of ATP was 1 mM. The average of three separate experiments is shown. The results are presented as a double-reciprocal plot of the concentration-response data. Additional experimental details are provided in the text. Standard error of the mean =  $\leq 15\%$ .

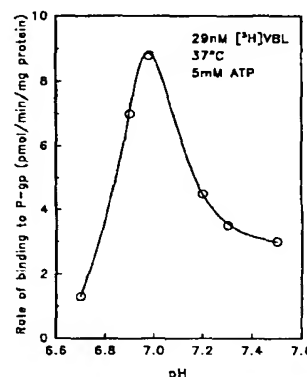


Fig. 8. The effect of pH on the initial phase of uptake of [ $^3$ H]VBL by inside-out vesicles from MEL/VCR-6 cells. The rate of uptake at 37  $^{\circ}$ C in the 0–1-min interval was determined. The [ $^3$ H]VBL concentration was 29 nM, and the ATP concentration was 5 mM. Additional experimental details are given in the text. The data shown are an average of three experiments done on separate days. Standard error of the mean =  $\leq 14\%$ .

vesicle compartment. The data shown in Fig. 9 represent calculations of the distribution ratio (intravesicular/extravesicular) in concentration of [ $^3$ H]VBL with respect to



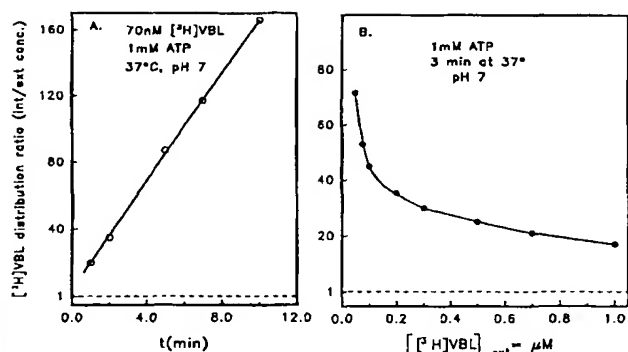


FIG. 9. Time and concentration dependence for ATP-dependent internalization of osmotically active  $[^3\text{H}]\text{VBL}$  by inside-out vesicles from MEL/VCR-6 cells. The data are presented as the distribution ratio (inside/outside) for osmotically active  $[^3\text{H}]\text{VBL}$  with respect to time (A) and concentration (B). The osmotically active fraction of intravesicular  $[^3\text{H}]\text{VBL}$  was determined from a comparison of ATP-dependent and non-ATP-dependent uptake at the indicated time and concentrations of  $[^3\text{H}]\text{VBL}$ . Additional experimental details are given in the text. The data are an average of three separate experiments. Standard error of the mean =  $\pm 12\%$ .

time during incubation of vesicles with 70 nM  $[^3\text{H}]\text{VBL}$  at 37 °C (Fig. 9A) and during a 3-min incubation at 37 °C with varying concentrations of  $[^3\text{H}]\text{VBL}$  (Fig. 9B). From this data, it can be seen that intravesicular accumulation of  $[^3\text{H}]\text{VBL}$  against a concentration gradient (Fig. 9) was evident by 1 min of incubation with 70 nM  $[^3\text{H}]\text{VBL}$  and continued to increase during the course of the incubation. Moreover, the distribution ratio exhibited (Fig. 9B) a hyperbolic downward relationship with extravesicular  $[^3\text{H}]\text{VBL}$ . This changing relationship with concentration is entirely consistent with the mediation of intravesicular accumulation by a saturable transport process.

**Effect of Various Agents and Protonophores on  $[^3\text{H}]\text{VBL}$  Accumulation by Inside-out Vesicles from MEL/VCR-6 Cells—**Since a delineation between P-gp binding and transport of  $[^3\text{H}]\text{VBL}$  was probably obtained in these studies, it was of interest to examine in more detail the manner by which some of the other identified inhibitors of P-gp function actually achieved their effect when compared to the effect of VRP in the same experiment. This could be easily visualized from a comparison of the time-course plots. For instance, although VRP ( $\text{IC}_{50} = 6 \pm 1 \mu\text{M}$ ), QND ( $\text{IC}_{50} = 11 \pm 2 \mu\text{M}$ ), RSP ( $\text{IC}_{50} = 14 \pm 2$ ), and orthovanadate ( $\text{IC}_{50} = 7 \pm 1 \mu\text{M}$ ) were all inhibitors of intravesicular accumulation of  $[^3\text{H}]\text{VBL}$ , the characteristics of this inhibition (see Fig. 10) were quite different. In the case of VRP, QND, and RSP there was an effect on the slope of the time-course plot for intravesicular accumulation of  $[^3\text{H}]\text{VBL}$ , but also a commensurate downward dislocation of the plot in relation to the origin similar to that shown for nonradioactive VBL in the same figure. In the case of orthovanadate, however, there was only a reduction in the slope of the time-course plot for accumulation like that seen with C219 antibody (Fig. 5B). These differences shown in this figure and in Figs. 3 and 4 are interpreted as reflecting a direct effect on binding of  $[^3\text{H}]\text{VBL}$  to P-gp by VRP, QND, and RSP, which have been shown (56–61) to competitively displace specific photoaffinity labels of P-gp. The effects of orthovanadate and C219 antibody appear to be on function, ostensibly at the level of permeant translocation or ATP hydrolysis. In this context, we also evaluated the effects of various protonophores on intravesicular accumulation of  $[^3\text{H}]\text{VBL}$ . These results show (data not given) that CFCCP at 0.5  $\mu\text{M}$  and 2,4-DNP at 50  $\mu\text{M}$  were ineffective or minimally effective as inhibitors of this process. The former was used at a concentration found to markedly affect proton gradients in mi-

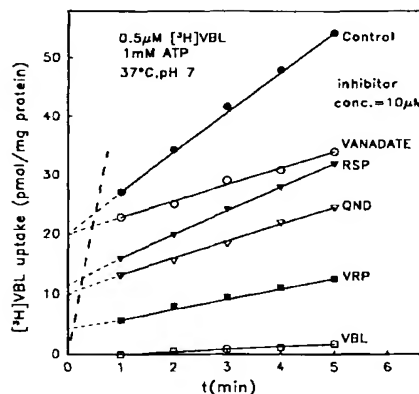


FIG. 10. Effect of various compounds on ATP-dependent intravesicular accumulation of  $[^3\text{H}]\text{VBL}$  by inside-out vesicles from MEL/VCR-6 cells. The individual compounds were added at the concentrations indicated in the figure. See text for additional details. The data are an average of three separate experiments. Standard error of the mean =  $\pm 14\%$ .

tochondria. Higher concentrations of CFCCP and of 2,4-DNP were not employed as they appeared to effect the binding of  $[^3\text{H}]\text{VBL}$  to P-gp as also indicated by the downward dislocation of the time-course plots (data not shown). We also showed that an inhibitor of  $\text{H}^+/\text{K}^+$  exchange, nigericin, was a strong inhibitor of intravesicular accumulation of  $[^3\text{H}]\text{VBL}$ . However, this inhibition was obtained (data not shown) in the absence of  $\text{K}^+$ . In this case, a downward dislocation of the time-course plot occurred, as in the case of VRP, QND and RSP in addition to a reduction of slope, suggesting as well an interaction at the level of  $[^3\text{H}]\text{VBL}$  binding.

#### DISCUSSION

Our results would appear to clarify the notion (4–11) of P-gp as a direct mediator of active, outwardly directed flux of, at least, the vinca alkaloids in multidrug-resistant cells. They provided evidence for binding of  $[^3\text{H}]\text{VBL}$  to P-gp and transport occurring tandemly as distinctly separate events during association of this agent with inside-out plasma membrane vesicles derived from MEL/VCR-6 cells expressing murine MDR 3 with the latter step representing the rate-limiting step. This evidence is based upon results that delineated a rapid osmotically insensitive phase and a slower internalization phase of uptake of  $[^3\text{H}]\text{VBL}$  by these vesicles that were MDR-specific. The former and the latter were markedly perturbed by the addition of VRP, QND, and RSP, but only the latter phase was perturbed by the addition of C219 anti-P-gp antibody and orthovanadate to the reaction system. The relative potency of VRP, QND, and RSP as inhibitors of the initial, rapid phase of uptake is similar to that shown (56–61) for their ability to competitively displace photoaffinity labels from P-gp during its detection by SDS-polyacrylamide gel electrophoresis. Although mediated transport of  $[^3\text{H}]\text{VBL}$  rather than its putative binding to P-gp was rate-limiting to net intravesicular accumulation, the value derived for apparent  $K_m$  for transport was very similar to the value derived for  $K_d$  for binding. Finally, the addition of a high concentration of nonradioactive VBL to the reaction system essentially eliminated both binding and transport phases of  $[^3\text{H}]\text{VBL}$  uptake by these vesicles. This would appear to suggest that virtually all of the intravesicular accumulation of  $[^3\text{H}]\text{VBL}$  was mediated by a saturable process and that in comparison there is very little simple diffusion of  $[^3\text{H}]\text{VBL}$  into these inside-

out vesicles. We also have derived<sup>2</sup> a value for the second-order rate constant,  $k_{on}$ , for this initial uptake phase and find it is in the range of  $1.5\text{--}2 \times 10^5 \text{ mol}^{-1} \text{ s}^{-1}$ . This value is clearly in the range of that expected for what is most likely a very complicated binding reaction between two highly complex structures such as VBL and P-gp.

Our ability to kinetically delineate separate rates for binding of [<sup>3</sup>H]VBL and its subsequent transport has allowed us to evaluate more incisively those factors that have been shown (4–7) to modulate the net accumulation of this agent in these vesicle systems. For instance, it was of interest to note that while apparent binding and transport of [<sup>3</sup>H]VBL were markedly temperature-dependent, only the latter exhibited any ATP dependence. Therefore, findings obtained here and elsewhere (26, 27, 31–33) showing that ATP could not be replaced in these inside-out vesicle systems by analogues such as ATP $\gamma$ S can now be interpreted in terms of a requirement by the transport (translocation) process *per se*, but not binding, for ATP hydrolysis. The same can be said for the well described (26, 27, 31–33) effect of the ATPase inhibitor, orthovanadate, and the anti-P-gp C219 antibody, which do not appear to affect binding of [<sup>3</sup>H]VBL to P-gp. Similarly, we have found that binding of [<sup>3</sup>H]VBL by P-gp exhibited a distinct pH dependence with an optimum in the neutral range. This was considerably different from that we have documented (36) for ATP-dependent folate analogue efflux in a similar manner where the pH optimum for the system, which was otherwise quite similar to P-gp, was in a much more acid range. In addition to the above, our findings can clearly be interpreted as documenting active, P-gp-mediated transport of [<sup>3</sup>H]VBL in this inside-out vesicle system. The magnitude of the gradient assumed by these inside-out vesicles, particularly at the lower end of the concentration-response range, was extremely large. This can be explained by the fact that the major route of exit of [<sup>3</sup>H]VBL from these MEL/VCR-6-derived vesicles in this orientation is severely compromised (see above). The implications of these results are important in that they provide direct proof of a property of P-gp that hitherto has been assumed or claimed, but in our opinion, not documented.

An alternative model for P-gp-mediated multidrug resistance views (18–20, 22) P-gp as an ion pump that only indirectly determines net accumulation of cytotoxic agents. This model incorporates the notion that P-gp maintains a pH or electrochemical gradient, which modulates partitioning by simple diffusion of these agents, most of which are weakly charged cations. This model is supported by findings (18–20, 22) that show that some MDR cells are pH elevated, have altered pH regulation and membrane potential, or can translocate chloride ions. Studies from other laboratories have provided evidence either in support of (24, 62, 63) or against (23, 34) the relevance of this model based on the effect of induced perturbations of pH or membrane potential on net accumulation of cytotoxic drugs.

Our own studies focused primarily on the issue of P-gp as a direct mediator of cytotoxic drug transport. These results, which we believe provide conclusive evidence for such a property, do not support or formally reject some role of pH or membrane potential alteration in determining net intravesicular accumulation of [<sup>3</sup>H]VBL in some multidrug-resistant cells. Indeed, these models need not be mutually exclusive. There is no documented evidence in the literature that is incompatible

with the notion that a membrane ATPase, which appears to transport so many structurally diverse agents, can also transport ions that may, in fact, represent its normal function. However, under the conditions employed here these vesicles do not appear to maintain a significant proton gradient, suggesting, at least, that P-gp is not a proton pump. Although, some of our results do tend to eliminate passive diffusion as contributing to intravesicular accumulation, an essential feature of a "pH model," this might merely reflect the absence of a significant pH or electrochemical gradient in this vesicle system. The lack of an appreciable effect of protonophores on transport was consistent with these findings. Clearly, further studies will be necessary on this issue before firm conclusions can be drawn. One obvious explanation for the diversity in results from studies that address this issue may be related to the different methodologies employed, and to the different pharmacologic and electrochemical properties and (or) lipophilicity of the various cytotoxic drugs that were used in each case.

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<sup>2</sup>  $k_{on}$  and  $k_{off}$  for putative binding of VBL were defined by the equation,  $d/dt B = k_{on}[B_T - B]L - k_{off}B$ , where  $B$  = amount bound/mg of vesicles,  $B_T$  = total P-gp/mg of vesicles, and  $L$  = VBL molar concentration.  $k_{on}$  and  $k_{off}$  are expressed in  $\text{mol}^{-1} \text{ s}^{-1}$ , and  $\text{s}^{-1}$ , respectively, and  $K_D = k_{off}/k_{on}$  [M] = half-saturation with VBL. Equilibrium time ( $T_{eq}$  at  $L = K_D$ ) was set at 20–30 s,  $T_{eq} = 1/k_{on}K_D + k_{off} = 1/2k_{off}$  at half-saturation.  $k_{on} = k_{off}/K_D = (1/20\text{--}30)(1/195) \times 10^5 \text{ mol} = 1.5\text{--}2 \times 10^5 \text{ mol}^{-1} \text{ s}^{-1}$ .

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